# INFLUENCE OF OMEGA-3 PUFAs ON THE METABOLISM OF PROANTHOCYANIDINS IN RATS

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**List of abbreviations**

EC, (epi)catechin

EGC, (epi)gallocatechin

Gluc, glucuronyl group

GSE, grape seed extract

Me, methyl group

MRM, multiple reaction monitoring

PAs, proanthocyanidins

STD, standard diet

Sulf, sulfate group

SPE, solid-phase extraction

**Abstract**

Studies of the bioavailability of proanthocyanidins usually consider them independently of other dietary constituents, while there is a tendency in the field of functional foods towards the combination of different bioactive compounds in a same product. This study examined the long-term effects of ω-3 polyunsaturated fatty acids of marine origin in the metabolic fate of grape proanthocyanidins. For this, female adult Wistar-Kyoto rats were fed (18 weeks) with a standard diet supplemented or not with eicosapentaenoic acid /docosahexanoic acid (1:1, 16.6 g/kg feed), proanthocyanidins-rich grape seed extract (0.8 g/kg feed) or both. A total of 39 microbial-derived metabolites and 16 conjugated metabolites were detected by HPLC-MS/MS either in urine or in the aqueous fraction of feces. An unexpected significant increase in many proanthocyanidin metabolites in urine and feces was observed in the ω-3 polyunsaturated fatty acids group as compared to the animals fed standard diet, which contained a small amount of polyphenols. However, proanthocyanidins metabolites in rats given ω-3 polyunsaturated fatty acids and grape seed extract did not significantly differ from those in the group supplemented only with grape seed extract. It was concluded that ω-3 polyunsaturated fatty acids collaborate in the metabolism of polyphenols when present at low doses, while the ability of ω-3 polyunsaturated fatty acids to induce microbiota transformations when proanthocyanidins are present at high doses is not relevant as compared to that of polyphenols themselves.

## 1. INTRODUCTION

Polyphenols are a large group of compounds found in plant foods that have been shown to have health-related effects in relation to several chronic diseases (Scalbert et al., 2005). Flavanols, included in the family of flavonoids, are among the most studied polyphenols. They range in complexity from monomers, such as (+)-catechin or (–)-epicatechin, to combinations of these structures via different linkages which gives rise to the corresponding oligomers or polymers- proanthocyanidins (PAs). Flavanols are found in many common foods, such as grapes, nuts or cocoa, and have been shown to have beneficial effects in relation to different markers of cardiovascular disease. Indeed, the European Food Safety Agency approved a health claim regarding the effects of cocoa flavanols on endothelium-dependent vasodilatation (EFSA, 2006).

A key aspect of the study of PAs is the proper knowledge of their metabolic fate, since they are extensively transformed after ingestion. Dimers and to a lesser extent trimers may be absorbed in the small intestine; the former may be methylated while no post-absorption transformation has been reported for trimers (Monagas et al., 2010). However, most ingested PAs reach the colon, being either extensively depolymerized and absorbed as monomers or metabolized by the gut microbiota (Touriño et al., 2011). Monomers are extensively conjugated in the liver and then circulate in the body before being excreted as urine or accumulated in tissues, or they return to the intestine via enterohepatic circulation. For those PA transformed by the microbiota, the resulting metabolites are mostly phenolic acids that may be absorbed and follow the same routes as polyphenols absorbed in the small intestine (Urpí-Sardá et al., 2009; Monagas et al., 2010; Mateos-Martín, Pérez-Jiménez, Fuguet, & Torres, 2012a). Increasing evidence suggests that circulating polyphenol-derived metabolites, especially those produced during colonic fermentation, may be the compounds responsible for the health-related properties of these food

85 constituents (Williamson, & Clifford, 2010).

86 In order to elucidate the metabolic fate of polyphenols, studies were designed based on
87 acute doses of these compounds alone (Urpí-Sardá et al., 2009; Monagas et al., 2010;
88 Touriño et al., 2011; Mateos-Martín et al., 2012a). In a common diet, however,
89 polyphenols are consumed in combination with other food components, which may have
90 either synergic or antagonistic effects on their bioavailability. Moreover, there is currently
91 increasing interest in the development of functional foods with combinations of bioactive
92 components (Peluso, Romanelli, & Palmery, 2014), which may affect the bioavailability of
93 polyphenols and the health effects derived from them. Therefore, now that the general
94 process of transformation of these compounds has been reported, there is increasing
95 interest in the effects that other food constituents, such as carbohydrates, proteins or dietary
96 fiber, may have on their bioavailability (Bohn, 2004; Zhang et al., 2014).

97 In relation to dietary fat, studies with animal models (Lesser, Cermak, & Wollfram, 2008)
98 and humans (Tulipani et al., 2002; Guo et al., 2013) have reported that this food constituent
99 increases the bioaccessibility and absorption of certain flavonoids, through different
100 mechanisms. Differential effects of long-chain and medium-chain fatty acids on the
101 bioavailability of polyphenols are probably due to the different metabolic routes that these
102 compounds follow (Lesser, Cermak, & Wollfram, 2006; Murota, Cermak, Terao, &
103 Wollfram, 2013). In contrast, the effects of fatty acids with different degree of unsaturation
104 on the metabolic fate of polyphenols have not been explored. Indeed, studies in this area
105 have only evaluated the differential effects of saturated and monounsaturated fats (Tulipani
106 et al., 2002; Lesser, Cermak, & Wollfram, 2006; Lesser, Cermak, & Wollfram, 2008; Guo
107 et al., 2013; Murota, Cermak, Terao, & Wollfram, 2013), and to the best of our knowledge,
108 only one *in vitro* study has considered the effects of a polyunsaturated fat: hazelnut oil
109 (Ortega, Macià, Romero, Reguant, & Motilva, 2011).

Long-chain ω-3 PUFAs of marine origin are a class of bioactive dietary components that have generated a great deal of interest due to their beneficial effects in both animal and human studies, on parameters related to cardiovascular disease (Aguilera, Díaz, Barcelata, Guerrero, & Ros, 2004; Lorente-Cebrián, Costa, Navas-Carretero, Zabala, Martínez, & Moreno-Aliaga, 2013). In a common diet, and in supplements containing different bioactive compounds, polyphenols may be consumed together with ω-3 PUFAs and different interactions may take place (Peluso, Romanelli, & Palmery, 2014), affecting also their metabolic fate. Therefore, the aim of this study was to evaluate the effect that ω-3 PUFAs had on the metabolic fate of grape PAs after long-term *in vivo* supplementation. To this end, a pilot study was carried out in Wistar-Kyoto rats, and the profile of polyphenol metabolites was measured by targeted HPLC-ESI-MS/MS analysis of urine and the aqueous fraction of feces.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and reagents

The standard diet was Teklad Global 2014 (Harlan Teklad Inc., Indianapolis, IN, USA). Fine Grajfnol® powder, 98% grape seed, was obtained from JF-Natural Product (Tianjin, China), with the following composition: total PAs (UV), ≥ 95%; oligomeric PAs, ≥ 60%; procyanidin dimer $B_2$ (HPLC), ≥ 1.8%; ash, ≤ 1.5%; weight loss on drying, ≤ 5.0%. Porcine gelatin type A 240/260 was from Juncà (Girona, Spain) and the soybean lecithin Topcithin 50 from Cargill (Barcelona, Spain). Oil with an eicosapentaenoic acid:docosahexaenoic acid (EPA:DHA) ratio of 1:1 was obtained by mixing appropriate quantities of the commercial fish oils AFAMPES 121 EPA (A.F.A.M.S.A., Vigo, Spain), EnerZona Omega 3 RX (Milan, Italy) and Oligen liquid DHA 80% (IFIGEN-EQUIP 98,

S.L., Barcelona). Soybean oil, obtained from unrefined organic soy oil (first cold pressing), was from Clearspring Ltd. (London, UK).

Ketamine chlorhydrate was purchased from Merial Laboratorios (Barcelona, Spain) and xylazine from Química Farmaceutica (Barcelona, Spain). Standards of (–)-epicatechin (≥ 98%), (–)-epigallocatechin (≥ 95%), 3-hydroxyphenylacetic acid (≥ 99%), 4-hydroxyphenylacetic acid (≥ 98%), 3,4-dihydroxyphenylacetic acid (≥ 98%), 3-hydroxybenzoic acid (≥ 99%), 4-hydroxybenzoic acid (≥ 99%), homovanillic acid (≥ 98%), vanillic acid (≥ 97%), caffeic acid (≥ 98%), 3,4-dihydroxyphenylpropionic acid (≥ 98%), 3-hydroxyphenylpropionic acid (≥ 98%), 4-hydroxyphenylpropionic acid (≥ 98%), 3,4-dihydroxybenzoic acid (≥ 97%), benzoic acid (≥ 99%), hippuric acid (≥ 98%), ferulic acid (≥ 99%), isoferulic acid (≥ 97%), *p*-coumaric acid (≥ 98%), *m*-coumaric acid (≥ 98%), gallic acid (≥ 97%), enterodiol (≥ 95%), phenylacetic acid (≥ 99%), taxifolin (≥ 85%), and tert-butylhydroquinone and formic acid (analytical grade) were obtained from Sigma Chemical (St Louis, MO, USA). Methanol (analytical grade) and hydrochloric acid (≥ 85%) were from Panreac (Castellar del Vallès, Barcelona, Spain). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Water for the assay solutions was obtained using a water Milli-Q purification system (Millipore Corporation, Billerica, MA, USA).

**2.2 Diets**

The 4 diets tested consisted of: the standard diet (STD group; $n$ = 5); the standard diet supplemented with ω-3 PUFAs (ω-3 group; $n$ = 5); the standard diet supplemented with grape seed extract (GSE group; $n$ = 5); and the standard diet supplemented with both ω-3 PUFAs and grape seed extract (ω-3+GSE group; $n$ = 5). All the diets were prepared in-house and included tert-butylhydroquinone as an antioxidant, porcine gelatin to promote gelatinization and soybean lecithin as an emulsifier. The mixture was freeze-dried to obtain

158 pellets that were stored at 4ºC to prevent oxidation and fungal contamination. The
159 composition of each diet, including the supplementations with ω-3 PUFAs and GSE, as
160 well as both the macronutrient and micronutrient profile, is shown in **Supplemental Table**
161 **1**. A mixture of EPA and DHA in a ratio 1:1 since was used since differential health effects
162 have been proposed for each fatty acid (Lorente-Cebrián et al. 2013). This ratio was
163 previously reported as the most beneficial one for cardiometabolic risk factors (Lluís et al.,
164 2013; Méndez et al., 2013). The diets without ω-3 PUFAs were enriched with soybean oil
165 in order to make them isocaloric. Fatty acid composition of the soybean oil and ω-3
166 PUFAs supplement, determined as methyl esters by gas chromatography (Lepage, & Roy,
167 1986), are provided as **Supplemental Table 2**. Because PUFAs are extremely susceptible
168 to oxidation and due to the potential toxic effects of lipid oxidation byproducts, the lipid
169 oxidation level was checked throughout the dietary interventional experiments (peroxide
170 values < 5 meq. oxygen per kg of oil).

171 The doses of ω-3 PUFAs (16.6 g/kg feed) and grape PAs (0.8 g/kg feed) were chosen
172 based on previous studies where similar doses showed beneficial effects (Masson et al.,
173 2008; Castell-Auví, Cedó, Pallarés, Blay, Pinent, & Ardévol., 2013) and because they
174 could be incorporated into a common diet.

175 **2.3 Animals and sample collection**

176 Twenty female, 8- to 9-week-old, Wistar-Kyoto rats (Charles River Laboratories,
177 Wilmington, MA, USA) were housed in cages ($n$ = 2-3/cage) under controlled conditions
178 of a 12 h light/12 h dark cycle, temperature of 22ºC ± 2ºC and relative humidity of 50% ±
179 10%. They had free access to water and pelleted feed (**Supplemental Table 1**) for 18
180 weeks after being randomly divided between the four dietary groups described above. For
181 urine and feces collection, the rats were individually placed in metabolic cages and

deprived of food for 24 h. At the end of the experiment, the rats were fasted overnight and anesthetized by an intraperitoneal injection of 80 mg/kg of ketamine (Imalgene 1000, Merial Laboratorios S.A., Barcelona, Spain) and 10 mg/kg of xylazine (Rompun 2%, Química Farmacéutica S.A., Barcelona, Spain).

The handling and killing of the animals were in full accordance with the European Union guidelines on the care and management of laboratory animals, and the pertinent permission was obtained from the CSIC Subcommittee for Bioethical Issues (ref. AGL2009-12 374-C03-03).

**2.4 Sample processing**

Urine samples were processed following a previously described solid-phase extraction (SPE) procedure (Touriño, Fuguet, Vinardell, Cascante, & Torres, 2009) for the extraction of phenolic metabolites. Briefly, Oasis HLB (60 mg) cartridges from Waters Corporation (Milford, MA, USA) were activated with 1 mL of methanol and 2 mL of water acidified to pH 3 with formic acid (acid water). Tthe samples (total volume of collected urine) were loaded, interfering components were removed with 9 mL of acid water and then the phenolic compounds were eluted with 1 mL of methanol. Taxifolin (final concentration of 5 mg $L^{-1}$) was used as internal standard.

Fecal samples collected over 24 h were re-suspended in acid water and homogenized in a vortex. Then, after adding the internal standard (taxifolin, 5 mg $L^{-1}$) the mixtures were centrifuged (10000 *g*, 10 min at 4ºC) to obtain a supernatant containing the aqueous fraction of the feces. This supernatant was freeze-dried and re-suspended in 1 mL of acid water, homogenized in a vortex, and then subjected to SPE and the work-up process described for the urine samples.

**2.5 HPLC-ESI-MS/MS analysis of polyphenol metabolites**

207 An Applied Biosystems (PE Sciex, Concord, Ontario, Canada) API 3000 triple quadrupole
208 mass spectrometer with a TurboIon spray source was used in negative mode to obtain MS
209 and MS/MS data. HPLC separations were performed on an Agilent 1100 series (Agilent,
210 Waldbronn, Germany) liquid chromatograph equipped with a Phenomenex (Torrance, CA,
211 USA) Luna C18 (50 x 2.0 mm i.d.) 3.0 μm particle size column and a Phenomenex
212 Securityguard C18 (4 x 2.0 mm i.d.) column. Gradient elution was performed with a binary
213 system consisting of [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in $CH_3CN$.
214 The following increasing linear gradient (v/v) of [B] was used, (t (min), % B): 0,8; 10,23;
215 15,50; 20,50; 21,100 followed by a re-equilibration step. MS conditions were as previously
216 described (Touriño, Fuguet, Vinardell, Cascante, & Torres, 2009). Each metabolite in the
217 urine samples was first identified by MRM (multiple reaction monitoring) transition of the
218 putative metabolites using a dwell time of 100 ms and then confirmed either by
219 comparison with a standard when available, second MRM, or neutral-loss and product ion
220 scan experiments- identification details were previously published (Molinar-Toribio et al.,
221 *in press*). The MS conditions for each MRM transition were optimized by direct injection
222 of metabolite standards, when available; for other metabolites, the conditions of the most
223 structurally similar standard were used. Analyst 1.4.2 software from AB Sciex was used for
224 data acquisition and processing. Standard calibrations curves were made for each
225 metabolite using between 4 and 11 different concentrations for each of them, between
226 0.001 and 60 mg $L^{-1}$, and they were used to determine the concentration of each metabolite
227 in the samples, after correction by the internal standard concentration. When no
228 commercial standard was available, the metabolites were quantified relatively using a
229 structurally related commercial standard- for details of the calibration curves used, see
230 **Supplemental Table 3**. The structurally related commercial standard may still show a

different response from that of the metabolite, so this method should be used mainly for comparative purposes.

### 2.6 Statistics

Results are expressed as mean concentrations ± Standard Error of the Mean (SEM), expressed in µmol/L urine or µmol/g dried feces, adjusted in both cases per kg feed/kg body weight. Mean body weight and feed consumption data per group were used for this adjustment. Since the data did not follow a normal distribution, the non-parametric Kruskal-Wallis and Mann-Whitney U tests were applied to analyze the significant differences ($P < 0.05$) comparing the four groups to one another. The Kruskal-Wallis test was applied to determine any significant difference between the treatments and, if any were detected, the Mann-Whitney U test was used to compare all the different pairs of the treatments. The SPSS IBM 19 package for Windows was used throughout.

## 3. RESULTS

### 3.1 Feed intake

Feed intake was monitored throughout the study. Mean values standardized by rat weight (g/kg rat/day) were: STD group, 59.4 (SEM 2.6); ω-3 group, 41.4 (SEM 4.5); GSE group, 56.4 (SEM 4.2); ω-3+GSE group, 40.0 (SEM 3.6). The intakes in ω-3 group and in the ω-3+GSE group were significantly lower than in the STD group (*P* 0.020 and 0.0058, respectively). The mean values of caloric intake (kcal/100 g rat/day) throughout the study were: STD group, 184.0 (SEM 38.9); ω-3 group, 128.4 (SEM 44.5); GSE group, 174.8 (SEM 44.5); ω-3+GSE group, 124.9 (SEM 39.7). Similarly, the energy intakes in ω-3 groups were significantly lower than in the STD and in the GSE groups. Due to these differences in the intakes, the results were adjusted per feed intake and body weight.

### 3.2 Conjugated metabolites of (epi)catechin and (epi)gallocatechin in urine

A total of 39 transitions were searched for in urine, corresponding to mono-, di- and tri-conjugated metabolites of (epi)catechin (EC) and (epi)gallocatechin (EGC) (derived from the combinations of the methylated or Me, sulfated or Sulf and glucuronidated or Gluc forms). The fragmentation patterns obtained for the different compounds were compared with those reported in the literature for PA-derived conjugated metabolites (Urpí-Sardá et al., 2009; Mateos-Martín et al., 2012a).

A total of 8 transitions were detected in the samples, corresponding to 12 metabolites from EC and 2 from EGC. Different metabolites may be identified by the same transition, since the substituent may be attached at different positions of the phenolic structure. This is the case, for example, of Gluc-EC, for which 5 different positional isomers were separated and identified by HPLC-MS/MS. The identification spectra for Gluc-EC is shown in **Supplemental Figure 1**. Characteristic fragments of this metabolite were detected at *m/z* 289, indicating the loss of the glucuronide moiety, as well as at *m/z* 175 and 113, corresponding to the degradation of this moiety.

The concentrations of the conjugated metabolites in urine are provided in **Table 1**. For the individual metabolites identified, urine concentrations of Gluc-EC-1, Me-Gluc-EC-1, Me-Gluc-EC-2 and Me-Gluc-EC-3 were significantly increased in both the GSE and ω-3+GSE groups, compared to the STD and ω-3 groups (Me-Gluc-EC-2, **Supplemental Figure 2)**. Gluc-EC-2 was also significantly increased in the GSE group but not in the ω-3+GSE group, compared to the STD group. In the ω-3 group, the urinary excretion of three glucuronidated EC forms was significantly higher than in the STD group.

Overall, the levels of conjugated metabolites of the monomers of PAs excreted in urine were increased in both the GSE and the ω-3+GSE groups as compared to the STD group.

### 3.3 Microbial-derived metabolites in urine

We searched for 48 transitions, corresponding to the microbial metabolites formed in the 8 different steps of the microbial fermentation of PAs (valerolactones, lignans, phenylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acids, cinnamic acids and glycinated benzoic acids) in the samples.

A total of 31 transitions were detected in the samples, corresponding to 39 metabolites, since it is known that some PAs microbial metabolites may present isomers - e.g., 3- or 4-hydroxybenzoic acid- and some of them may later be conjugated at different positions (Redeuil et al., 2011). For instance, the identification of *m*-coumaric and p-coumaric acid, with the same transition, was based on standard retention times (**Supplemental Figure 3**). Metabolite concentrations are shown in **Table 2**.

In the GSE group, 24 metabolites occurred at concentrations significantly greater than in the STD group. In the case of the ω-3+GSE group, this affected 19 metabolites. No significant differences were observed between the two groups supplemented with GSE. In the ω-3 group, the concentration of 18 metabolites significantly increased with respect to the STD group- in most of the cases, the concentrations found in this group were lower than those found in the GSE and in the ω-3+GSE group.

### 3.4 Microbial-derived metabolites in feces

None of the EC or EGC conjugated metabolites found in urine were detected in feces. However, 11 microbial-derived metabolites were identified in the fecal samples (**Table 3**). In the GSE and ω-3+GSE groups, 7 metabolites were significantly greater than in the STD group. 4-Hydroxyphenylpropionic acid was also significantly higher in the ω-3+GSE group as compared to the STD group. In the ω-3 group, 5 metabolites were at significantly greater concentrations than in the STD group. The concentrations in the ω-3 and the ω-3+GSE groups were in the same range for some compounds, while 4-hydroxyphenylpropionic acid showed the highest concentration in the ω-3+GSE group,

304 and 3-hydroxyphenylpropinic acid, Me-hippuric acid-1 and Me–hippuric acid-2 showed
305 the highest concentrations in the GSE group- for Me-hippuric acid-1 the increment was not
306 statistically significant .

307 **4. DISCUSSION**

308 Here, we carried out a pilot study on the effects of ω-3 PUFAs on the metabolic fate of
309 PAs; an aspect that had not previously been explored. To mimic a human dietary situation,
310 this was evaluated in rats after long-term exposure to diets that incorporated both
311 components. PA-derived conjugated EC and microbially generated metabolites were
312 measured in urine; the biological fluid considered the most appropriate for evaluating the
313 bioavailability of polyphenols (Pérez-Jiménez et al., 2010), given the short half-life in
314 plasma of their metabolites. These metabolites were also measured in fecal water, *i.e.*, the
315 fraction of the feces closest to the colonic epithelium (Gill et al., 2010) that could
316 potentially be involved in the reported effects of PAs on colonic health (Rossi, Bossetti,
317 Negri, Lagiou, & La Vecchia, 2010; Sánchez-Tena et al., 2013). Since intakes were
318 significantly different between the groups- i-e., the lowest intakes were found in the ω-3
319 groups- metabolite concentrations were adjusted per feed intake. Nevertheless, metabolite
320 concentrations in the GSE groups were in the same range as those reported in studies with
321 similar supplementations for shorter periods (Tsang et al., 2005; Choy et al., 2014). Also,
322 the nature of the metabolites found was the same that those previously reported in studies
323 on the metabolic fate of PAs, a process described in detail elsewhere (Mosele, Macià, &
324 Motilva, 2015). This shows that the same tendencies are maintained for long-term
325 supplementation.

326 We obtained an unexpected result, in that microbial-derived PA metabolites increased (in
327 urine and in feces), as did some EC conjugates, in the ω-3 PUFAs group (without GSE
328 supplementation). Since these metabolites are not present in the metabolic routes of ω-3

329 PUFAs, it seems that ω-3 PUFAs collaborated in the transformation of polyphenols already
330 present in the basic STD diet (and responsible for the values obtained in the STD group).
331 The STD diet contained wheat middlings, ground wheat and ground corn; cereals that
332 contain PAs among their phenolic compounds (McCallum, & Walker, 1990; Hichem,
333 Mounir, & Naceur, 2009; Arranz, & Saura-Calixto, 2010). Indeed, they contain not only
334 the PAs that occur free in the food matrix (known as extractable PAs), but also the fraction
335 associated with the dietary matrix, the so-called non-extractable PAs (Arranz, & Saura-
336 Calixto, 2010) which are also extensively metabolized by the colonic microbiota after
337 being released from the food matrix (Mateos-Martín, Pérez-Jiménez, Fuguet, & Torres,
338 2012b). Based on our results with the ω-3 PUFAs group, these fatty acids may promote PA
339 metabolism through interactions with the gut microbiota. This point needs to be addressed
340 further, as the information available to date is quite limited. Anyway, it seems that fish oil
341 supplementation increases the proportion of *Lacotobacilliales*, as observed in gnotobiotic
342 piglets, where there was an increase in *Lactobacillus paracasei* adhesion to the jejunal
343 mucosa (Bomba, Nemcová, Gankarciková, Herich, Guba, & Mudronová, 2002), an animal
344 model of colorectal cancer (Piazzi et al., 2014;) and rats with intestinal chronic rejection
345 (Li, Zhang, Wang, Tang, Zhang, & Li, 2011). Conversely, this kind of supplementation to
346 animal models originated a decrease in *Escherichia coli*, *Bacteroides* spp. and *Clostidriales*
347 spp., among others (Li et al., 2011; Yu, Zhu, Pan, Shen, Shan, & Das, 2014). Interestingly,
348 *Lactobacillus plantarum* has been reported to stimulate the colonic fermentation of red
349 wine polyphenols (Barroso et al., 2014), which are quite similar to the PA included in the
350 GSE tested here.
351 In the present study, STD diet had a soy oil content similar to that of PUFAs in the ω-3
352 PUFAs diet, in order to make them isocaloric. Since the main constituent of soy oil are
353 monounsaturated fatty acids (followed by PUFAs), the observed changes in the microbial

354 metabolism of PAs would also indicate differences in the microbiota profile when
355 consuming monounsaturated or polyunsaturated fats. Indeed, when comparing the effects
356 on human gut microbiome of three kind of monounsaturated fats with those of two kinds of
357 polyunsaturated fats, a consistency was observed between the modifications in bacteria
358 profile originated by monounsaturated fats and those derived from PUFAs consumption
359 (Pu, Khazenehei, Jones, & Khapifaur, 2016).
360 Additionally, since cereals contain phenolic acids as major phenolic compounds, and these
361 generate several metabolites in common with those of PAs (Rodríguez-Mateos et al.,
362 2014), it seems plausible that ω-3 PUFAs would also stimulate the release and
363 transformation of such compounds. Additionally, some metabolites, such as hippuric or
364 valerolactones, may originate from other food components (Pero, 2010; Molinar-Toribio et
365 al., *in press)*, so their increase in the ω-3 group might be due to an effect on other
366 metabolic routes, becoming particularly evident in a long-term study such as this one.
367 Another interesting result of this study was that, while GSE contained mostly (95%) PAs
368 (i.e., oligomers and polymers), in the groups supplemented with it there was an increase in
369 the monomeric conjugated metabolites of EC. This agrees with our previous suggestion
370 (Pérez-Jiménez et al., 2010) of a depolymerization of PAs by bacteria, releasing free EC
371 which would then be subjected to further absorption and conjugation. ω-3 PUFAs also
372 seem to affect the activity of the bacteria responsible for this, since in the ω-3 groups there
373 was also a tendency towards an increase in these compounds, as compared to the STD
374 group.
375 When GSE and ω-3 PUFAs were administered together, the concentrations of the detected
376 metabolites were in the same range than those found in the GSE group, without significant
377 differences. Therefore, ω-3 PUFAs did not have either an enhancing or inhibitory effect on
378 a diet supplemented with grape PA, despite the enhancing effects they showed towards the

379 transformation of polyphenols already present in the basal diet. It is known that polyphenol
380 supplementation causes a shift in microbial communities towards those species indeed able
381 to transform them, e.g. the *Eubacterium rectale* group (Selma, Espín, & Tomás-Barberán,
382 2009; Queipo-Ortuño et al., 2012). We hypothesize that the changes that ω-3 PUFAs may
383 cause in the microbiota, and that had an effect in the transformation of polyphenols already
384 present in the basal diet, may not be relevant against the modifications that polyphenols
385 themselves cause in the microbiota when provided at high doses.
386 Anyway, the concentration values for the pool of putatively beneficial circulating PA-
387 derived metabolites in the ω-3+GSE group were in the same range than in the GSE group.
388 So, proanthocyanidin metabolites from GSE are bioavailable for possible collaborative
389 functional effects with ω-3 PUFAs. Indeed, when evaluating the effects of ω-3 PUFAs and
390 GSE on the metabolic alterations induced by a high-fat high-sucrose diet, it was observed
391 that the combination was more efficient than the separate supplements at averting
392 metabolic alterations (Ramos-Romero et al., 2016).
393 The main limitations of this study are intrinsic to experiments on the metabolic fate of
394 polyphenols. First, there is a lack of commercial standards for many metabolites (Kay,
395 2010), which forced us to express the results as equivalents of the most closely related
396 compound, with an associated error. Secondly, we had to deal with the high inter-
397 individual variability of results; an aspect widely reported for the metabolic fate of
398 polyphenols in both animals and humans (Choy et al., 2014; Muñoz-González et al., 2014).
399 This latter aspect may have been exaggerated in this study, given that it involved long-term
400 supplementation, where the measured concentrations for each metabolite did not
401 correspond to the maxima and it is not known when each animal received the last dose of
402 polyphenols before fasting, since they were fed *ad libitum*. Also, higher number of animals
403 would have strengthened the statistical significance of the differences detected in some

metabolites. Be that as it may, this did not preclude us from observing the emergence of some general tendencies, as discussed above; while at the same time, it has the advantage of reflecting a situation closer to a genuine human dietary situation.

## 5. CONCLUSIONS

This study shows that combined long-term supplementation with ω-3 PUFAs and PAs from GSE to healthy rats did not significantly affect the levels of urinary and fecal PA metabolites, compared with supplementation with GSE alone. Meanwhile, ω-3 fatty acids seem to encourage the metabolism of the polyphenols present in the STD feed. Briefly, ω-3 PUFAs appear to collaborate in the release and metabolism of polyphenols when they are present at low doses, while at high doses their ability to induce transformations does not seem to be relevant as compared to that of polyphenols themselves.

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## AUTHORS' CONTRIBUTIONS

I.M., J.L.T. and J.P.-J. designed the research. E.M.-T., S.R.-R., N.T., M.R., L.M., and J. P.-J. conducted the research. E.M.-T., S.R.-R., E.F. and J.P.-J. analyzed the data. J.P.-J. and

J.L.T. wrote the first draft of the manuscript. All the authors contributed to writing the manuscript and approved the final version. J.P.-J. and J.L.T. had primary responsibility for final content.

## CONFLICT OF INTEREST

None of the authors declare any conflict of interest.

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## TABLES

**Table 1.** (Epi)catechin and (epi)gallocatechin conjugated metabolites in urine from rats fed a standard diet without supplementation (STD) or supplemented with ω-3 PUFAs (ω-3), grape seed extract (GSE) or ω-3 PUFAs and grape seed extract (ω-3 + GSE)[1]. Results expressed as μM, adjusted per kg feed intake/kg body weight, after quantification with structurally similar commercial standards (see Table S3).

| Metabolite | STD[2] | | ω-3 | | | GSE[2] | | | ω-3 + GSE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | *P* | Mean | SEM | *P* | Mean | SEM | *P* |
| *EC monoconjugated* | | | | | | | | | | | |
| Gluc-EC-1 | n.d. | | n.d. | | | 5.68 | 2.87 | 0.0081[a, b] | 6.04 | 5.21 | 0.0081[a, b] |
| Gluc-EC-2 | n.d. | | n.d. | | | 3.84 | 1.93 | 0.0321[a, b] | 3.29 | 3.18 | |
| Gluc-EC-3 | 0.64 | 0.32 | 8.00 | 1.40 | 0.0081[a] | 3.09 | 1.21 | | 15.55 | 5.37 | 0.0081[a] |
| | | | | | | | | | | | 0.0321[b] |
| Gluc-EC-4 | 0.32 | 0.09 | 2.83 | 0.75 | 0.0081[a] | 0.95 | 0.47 | | 4.49 | 1.60 | 0.0081[a] |
| | | | | | | | | | | | 0.0321[c] |
| Gluc-EC-5 | 0.88 | 0.44 | 3.67 | 0.80 | 0.0321[a] | 1.96 | 1.03 | | 6.34 | 1.86 | 0.0161[a] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Total | 1.84 | 0.61 | 14.50 | 2.86 | 0.0081[a] | 15.52 | 6.24 | 0.0161[a] | 35.72 | 16.64 | 0.0081[a] |
| *EC diconjugated* | | | | | | | | | | | |
| Gluc-Sulf-EC | 0.97 | 0.40 | 1.05 | 0.20 | | 3.54 | 1.62 | 0.0321[b] | 2.99 | 1.17 | |
| Me-Gluc-EC-1 | n.d. | | n.d. | | | 0.85 | 0.28 | 0.0081[a,b] | 1.39 | 0.98 | 0.0081[a,b] |
| Me-Gluc-EC-2 | n.d. | | n.d. | | | 14.79 | 4.88 | 0.0081[a,b] | 13.61 | 9.21 | 0.0081[a,b] |
| Me-Gluc-EC-3 | n.d. | | n.d. | | | 5.26 | 1.68 | 0.0081[a,b] | 5.79 | 4.10 | 0.0081[a,b] |
| Me-Sulf-EC | 2.46 | 0.84 | 2.00 | 0.32 | | 3.03 | 0.58 | 0.0081[a,b] | 3.85 | 1.27 | 0.0161[a] |
| | | | | | | | | | | | 0.0081[b] |
| Total | 3.43 | 1.23 | 3.05 | 0.47 | | 27.47 | 8.09 | 0.0081[a,b] | 27.64 | 16.33 | 0.0161[a] |
| | | | | | | | | | | | 0.0081[b] |
| *EC triconjugated* | | | | | | | | | | | |
| 3Me-EC | 1.09 | 0.27 | 0.94 | 0.17 | | 1.07 | 0.30 | | 1.65 | 0.48 | |
| 2Me-Gluc-EC | 0.63 | 0.23 | 0.86 | 0.29 | | 1.51 | 0.37 | | 1.73 | 0.75 | |
| Total | 1.72 | 0.47 | 1.80 | 0.46 | | 2.58 | 0.58 | | 3.38 | 1.17 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *EGC diconjugated* | | | | | | | | |
| 2Sulf-EGC | 14.16 | 2.81 | 15.55 | 2.71 | 21.69 | 4.18 | 27.72 | 8.75 |
| *EGC triconjugated* | | | | | | | | |
| Me-Gluc-Sulf-EGC | 3.95 | 1.23 | 8.71 | 1.29 | 8.17 | 0.98 | 16.17 | 5.95 |

n.d., non-detected; Gluc, glucuronide; Me, methyl; Sulf, sulfated

[1] Values are mean ± SEM , *n*=5

[2] Molinar-Toribio et al., *in press*.

[a] differences with respect to STD group; [b] differences with respect to ω-3 group; [c] differences with respect to GSE group. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney U tests; pairs comparisons were performed between all the groups.

**Table 2.** Microbial-derived proanthocyanidin metabolites in urine from rats fed a standard diet without supplementation (STD) or supplemented with ω-3 PUFAs (ω-3), grape seed extract (GSE) or ω-3 PUFAs and grape seed extract (ω-3 + GSE) [1]. Results expressed as μM, adjusted per kg feed intake/kg body weight , after quantification with structurally similar commercial standards (see Table S3).

| Metabolite | STD[2] | | ω-3 | | | GSE[2] | | | ω-3 + GSE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | *P* | Mean | SEM | *P* | Mean | SEM | *P* |
| *Valerolactones* | | | | | | | | | | | |
| 3- or 4-Hydroxyphenylvalerolactone | 24.17 | 7.33 | 205.81 | 15.67 | 0.0081[a] | 379.24 | 129.69 | 0.0081[a] | 604.60 | 268.55 | 0.0081[a] |
| 3,4-Dihydroxyphenylvalerolactone | 5.97 | 4.09 | 89.81 | 26.91 | 0.0081[a] | 219.53 | 73.37 | 0.0081[a] | 595.09 | 281.69 | 0.0081[a] |
| Gluc-3,4-dihydroxyphenylvalerolactone | 51.48 | 15.61 | 133.16 | 41.14 | | 164.87 | 29.47 | 0.0081[a] | 318.25 | 115.12 | 0.0321[a] |
| Sulf-3,4-dihydroxyphenylvalerolactone | 15.92 | 9.76 | 535.46 | 98.76 | 0.0081[a] | 1373.86 | 117.59 | 0.0081[a,b] | 1638.69 | 604.44 | 0.0081[a] |
| 3-Hydroxyphenylmethylvalerolactone | 24.17 | 7.20 | 32.31 | 4.5 | | 93.35 | 26.46 | 0.0081[a] | 56.64 | 16.46 | |
| | | | | | | | | 0.0321[b] | | | |
| 4-Hydroxyphenylmethylvalerolactone | 214.92 | 73.50 | 341.12 | 59.26 | | 628.27 | 174.41 | 0.0321[a] | 481.06 | 125.84 | |
| Gluc-3-hydroxymethylphenylvalerolactone | 112.73 | 42.56 | 128.90 | 58.41 | | 171.21 | 23.38 | 0.0321[b] | 170.88 | 57.94 | |
| Sulf-3- or 4-hydroxymethylphenylvalerolactone | 69.40 | 23.06 | 37.77 | 7.81 | | 91.63 | 16.42 | | 77.34 | 22.49 | |
| Total | 517.06 | 103.86 | 1504.34 | 305.22 | 0.0081[a] | 3121.97 | 446.61 | 0.0081[a] | 3987.54 | 1393.77 | 0.0081[a] |
| | | | | | | | | 0.0321[b] | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Lignans* | | | | | | | | | | | |
| Enterolactone | >60 | | >60 | | | >60 | | | >60 | | |
| Sulf-enterolactone | >60 | | >60 | | | >60 | | | >60 | | |
| *Phenylvaleric acids* | | | | | | | | | | | |
| 3-Hydroxyphenylvaleric acid | 31.10 | 13.42 | 20.63 | 5.25 | | 102.08 | 15.47 | 0.0161[a] 0.0081[b] | 157.89 | 73.49 | 0.0321[b] |
| 4-Hydroxyphenylvaleric acid | 4.63 | 1.71 | 17.37 | 3.74 | 0.0321[a] | 47.35 | 15.97 | 0.0081[a] | 28.53 | 4.43 | 0.0081[a] |
| 3,4-Dihydroxyphenylvaleric acid | 8.74 | 2.49 | 70.33 | 18.25 | 0.0081[a] | 47.14 | 12.24 | 0.0161[a] | 86.66 | 22.09 | 0.0081[a] |
| Sulf-3,4-dihydroxyphenylvaleric acid | 28.02 | 11.34 | 530.70 | 160.71 | 0.0081[a] | 1053.66 | 220.98 | 0.0081[a] | 1061.01 | 403.49 | 0.0081[a] |
| Total | 72.49 | 23.05 | 639.03 | 182.16 | 0.0081[a] | 1250.23 | 223.86 | 0.0081[a] | 1334.09 | 482.74 | 0.0081[a] |
| *Phenylpropionic acids* | | | | | | | | | | | |
| 3-Hydroxyphenylpropionic acid | 6638.51 | 2746.72 | 4097.84 | 597.18 | | 14257.84 | 9274.35 | | 8957.36 | 2307.88 | |
| 4-Hydroxyphenylpropionic acid | >60 | | >60 | | | >60 | | | >60 | | |
| Gluc-3- or- 4hydroxyphenylpropionic acid | 21.34 | 12.93 | 13.93 | 6.64 | | 17.10 | 2.09 | | 28.36 | 11.52 | |
| Dihydrocaffeic acid (3,4-Dihydroxyphenylpropionic acid) | 3.65 | 1.60 | 10.49 | 2.65 | | 56.85 | 45.95 | 0.0321[a] | 20.82 | 5.86 | |
| Sulf-3,4-dihydrocaffeic acid | 39.65 | 15.31 | 41.14 | 10.75 | | 120.60 | 76.86 | | 60.74 | 15.85 | |
| Total[2] | 670.47 | 2751.81 | 4163.41 | 610.83 | | 14452.40 | 9397.29 | | 9067.28 | 2334.52 | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Phenylacetic acids* | | | | | | | | | | | |
| 3-Hydroxyphenylacetic acid | 63.14 | 26.56 | 324.13 | 67.53 | 0.0081[a] | 427.27 | 67.29 | 0.0081[a] | 838.83 | 284.14 | 0.0321[a] |
| 4-Hydroxyphenylacetic acid | 60.97 | 23.56 | 532.53 | 142.90 | 0.0081[a] | 1816.39 | 312.24 | 0.0081[a] | 1859.17 | 585.26 | 0.0081[a] |
| | | | | | | | | 0.0321[b] | | | |
| 3,4-Dihydroxyphenylacetic acid | 0.86 | 0.36 | 3.09 | 0.61 | 0.0161[a] | 10.27 | 5.04 | 0.0081[a] | 16.65 | 4.87 | 0.0161[a] |
| Sulf-3,4-dihydroxyphenylacetic acid | 7.79 | 4.41 | 8.91 | 1.48 | | 8.41 | 1.90 | | 14.19 | 4.19 | |
| Total | 132.77 | 46.12 | 868.66 | 170.81 | 0.0081[a] | 2262.34 | 292.60 | 0.0081[a] | 2728.84 | 745.56 | 0.0081[a] |
| | | | | | | | | 0.0161[b] | | | |
| *Benzoic acids* | | | | | | | | | | | |
| 4-Hydroxybenzoic acid | 13.88 | 5.56 | 78.01 | 23.75 | 0.0081[a] | 63.86 | 20.04 | 0.0321[a] | 52.31 | 13.40 | |
| 3,4-Dihydroxybenzoic acid | 0.36 | 0.22 | 7.16 | 2.72 | 0.0161[a] | 21.35 | 9.80 | 0.0081[a] | 19.36 | 11.58 | 0.0081[a] |
| Gluc-3-hydroxybenzoic acid | 0.24 | 0.13 | 1.04 | 0.34 | | 2.73 | 1.17 | 0.0081[a] | 1.77 | 0.50 | 0.0321[a] |
| Gluc-4-hydroxybenzoic acid | 0.02 | 0.01 | 0.14 | 0.03 | 0.0321[a] | 0.31 | 0.09 | 0.0081[a] | 0.26 | 0.17 | 0.0321[a] |
| Sulf-3,4-dihydroxybenzoic acid | 6.52 | 2.08 | 20.11 | 3.45 | 0.0081[a] | 69.10 | 37.61 | 0.0081[a] | 34.97 | 9.90 | 0.0321[a] |
| Sulf-vanillic-acid | 327.72 | 64.53 | 681.32 | 200.67 | | 459.50 | 78.29 | | 1432.01 | 507.86 | |
| Total | 348.74 | 60.23 | 787.78 | 228.73 | | 616.85 | 129.27 | 0.0161[a] | 1540.69 | 539.51 | |
| *Cinnamic acids* | | | | | | | | | | | |
| Caffeic acid | 0.95 | 0.44 | 1.18 | 0.74 | | 3.07 | 1.85 | | 1.39 | 0.45 | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *m*-Coumaric acid | 116.69 | 54.97 | 102.91 | 29.16 | | 247.53 | 30.90 | | 140.64 | 47.78 | |
| *p*-Coumaric acid | 24.03 | 8.90 | 20.41 | 10.93 | | 32.90 | 9.37 | | 46.87 | 31.48 | |
| Sulf-coumaric acid-1 | n.d. | | 6.53 | 4.78 | 0.0081[a] | 14.05 | 4.77 | 0.0081[a] | 7.67 | 5.87 | 0.0081[a] |
| Sulf-coumaric acid-2 | 0.04 | 0.01 | 7.00 | 5.09 | 0.0321[a] | 13.37 | 4.17 | 0.0081[a] | 7.53 | 5.76 | 0.0081[a] |
| Ferulic acid | 15.31 | 6.21 | 18.23 | 15.20 | | 20.30 | 7.54 | | 29.46 | 14.32 | |
| Total | 157.02 | 65.75 | 156.26 | 65.01 | | 331.22 | 44.11 | | 235.56 | 101.24 | |
| *Glycinated benzoic acids* | | | | | | | | | | | |
| Hippuric acid | 48.02 | 17.74 | 1385.71 | 396.40 | 0.0081[a] | 2219.53 | 1311.56 | 0.0081[a] | 1494.77 | 317.90 | 0.0081[a] |
| Hydroxyhippuric acid | 0.29 | 0.26 | 14.31 | 5.03 | 0.0081[a] | 16.99 | 3.57 | 0.0081[a] | 18.23 | 5.35 | 0.0081[a] |
| Me-hippuric acid-1 | 0.15 | 0.15 | 67.03 | 54.54 | 0.0081[a] | 94.37 | 35.88 | 0.0081[a] | 73.06 | 57.30 | 0.0081[a] |
| Me-hippuric acid-2 | 2.91 | 1.22 | 5.49 | 4.84 | | 14.05 | 7.41 | | 16.62 | 13.76 | |
| Total | 51.37 | 18.50 | 1472.53 | 443.46 | 0.0081[a] | 2344.94 | 1313.91 | 0.0081[a] | 1602.68 | 328.88 | 0.0081[a] |

n.d., non-detected; Gluc, glucuronide; Me, methyl; Sulf, sulfated

Compounds detected in all the groups above the highest concentration of the calibration: Enterolactone, Sulf-Enterolactone and 4-Hydroxyphenylpropionic acid

[1] Values are mean ± SEM , *n*=5

[2] Molinar-Toribio et al., *in press.*

[a] differences with respect to STD group; [b] differences with respect to ω-3 group. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney U tests; pairs

comparisons were performed between all the groups.

**Table 3.** Microbial-derived proanthocyanidin metabolites in feces from rats fed a standard diet without supplementation (STD) or supplemented with ω-3 PUFAs (ω-3), grape seed extract (GSE) or ω-3 PUFAs and grape seed extract (ω-3 + GSE)[1]. Results expressed as µmol/ g dried faeces, adjusted per kg feed intake/kg body weight, after quantification with structurally similar commercial standards (see Table S3).

| Metabolite | STD[2] | | ω-3 | | | GSE[2] | | | ω-3 + GSE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | *P* | Mean | SEM | *P* | Mean | SEM | *P* |
| *Lignans* | | | | | | | | | | | |
| Enterolactone | > 60 | | > 60 | | | > 60 | | | > 60 | | |
| *Phenylvaleric acids* | | | | | | | | | | | |
| 3-Hydroxyphenylvaleric acid | 3.14 | 1.47 | 78.48 | 36.55 | 0.0161[a] | 985.26 | 872.17 | 0.0081[a] | 975.65 | 485.91 | 0.0081[a] |
| *Phenylpropionic acids* | | | | | | | | | | | |
| 3-Hydroxyphenylpropionic acid | 45.05 | 3.84 | 35.68 | 23.50 | 0.0161[a] | 101.96 | 93.02 | 0.0081[a] | 12.60 | 0.00 | 0.0081[a] |
| 4-Hydroxyphenylpropionic acid | 10.91 | 2.77 | 381.06 | 242.99 | 0.0321[a] | 41.15 | 32.21 | | 2033.79 | 1653.65 | 0.0321[a] |
| Total | 55.96 | 41.61 | 416.73 | 246.22 | | 143.10 | 90.51 | | 2046.39 | 1653.65 | |
| *Benzoic acids* | | | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4-hydroxybenzoic acid | n.d. | | 0.15 | 0.10 | | 1.06 | 0.75 | 0.0081[a] | 3.94 | 1.68 | 0.0081[a] |
| 3,4-Dihydroxybenzoic acid | n.d. | | n.d. | | | 0.22 | 0.16 | 0.0081[a,b] | 0.26 | 0.14 | 0.0321[a, b] |
| Total | n.d. | | 0.15 | 0.10 | | 1.27 | 0.91 | 0.0081[a] | 4.20 | 1.72 | 0.0081[a] |
| *Cinnamic acids* | | | | | | | | | | | |
| Caffeic acid | 0.01 | 0.01 | 0.51 | 0.29 | 0.0081[a] | 0.53 | 0.41 | 0.0081[a] | 1.55 | 0.67 | 0.0081[a] |
| *p*-coumaric acid | 0.04 | 0.02 | 1.66 | 1.02 | | 2.72 | 2.06 | 0.0321[a] | 4.83 | 1.76 | 0.0321[a] |
| Total | 0.05 | 0.03 | 2.16 | 1.31 | 0.0321[a] | 3.25 | 2.47 | 0.0321[a] | 6.38 | 2.41 | 0.0161[a] |
| *Glycinated benzoic acids* | | | | | | | | | | | |
| Hippuric acid | 0.05 | 0.03 | 0.75 | 0.75 | | 0.04 | 0.03 | | 1.50 | 0.76 | |
| Me-hippuric acid-1 | 6.54 | 5.23 | 486.95 | 453.35 | | 12279.53 | 11463.14 | | 1023.35 | 584.48 | |
| Me-hippuric acid-2 | n.d. | | 269.32 | 165.44 | 0.0081[a] | 6283.37 | 5735.59 | 0.0081[a] | 137.49 | 119.35 | 0.0081[a] |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Total | 6.59 | 5.24 | 757.01 | 567.91 | 18562.94 | 17211.60 | 0.0081[a] | 1162.33 | 554.38 | 0.0081[a] |

n.d., non-detected; Gluc, glucuronide; Me, methyl; Sulf, sulfated

[1] Values are mean ± SEM , *n*=5

[2] Molinar-Toribio et al., *in press.*

[a] differences with respect to STD group; [b] differences with respect to ω-3 group. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney U tests; pairs comparisons were performed between all the groups.